ANNALS OF SURGERY
Vol. 229, No. 3, 385–392

Phospholipase A_2 Mediates Nitric Oxide Production by Alveolar Macrophages and Acute Lung Injury in Pancreatitis

Yasuhiro Tsukahara, MD, Takashi Morisaki, MD, Yoshiyuki Horita, MD, Motomichi Torisu, MD, and Masao Tanaka, MD

From the Division of Clinical Immunology, Department of Surgery 1, Kyushu University, Faculty of Medicine, Fukuoka, Japan

Objective

Reportedly, nitric oxide (NO) derived from alveolar macrophages (AMs) and increased serum phospholipase A_2 (PLA_2) activity are associated with the pathogenesis of lung injury in acute pancreatitis. The authors examined the possibility that PLA_2 causes, in part, the induction of NO production by AMs in pancreatitis.

Methods

Pancreatitis was induced in rats by selective pancreatic duct ligation (SPL). AMs were stimulated with PLA_2 or SPL rat serum, with or without administration of the PLA_2 inhibitor quinacrine. Then NO production from the AMs was measured by the Griess method, inducible NO synthase mRNA expression of AMs was analyzed by the reverse transcription–polymerase chain reaction, and cytotoxic effects of AMs on human umbilical vein endothelial cells was examined by a ^{51}Cr release assay. *In vivo,* the effect of quinacrine on lung injury was determined by measuring the arterial blood oxygen pressure (Pao_2), lung weight, and lung permeability using Evans blue dye concentration of SPL rat.

Results

In vitro, the serum with high PLA_2 activity induced NO production by rat AMs. PLA_2 (50 ng/ml) induced significant amounts of NO production, inducible NO synthase mRNA expression, and cytotoxicity toward the human umbilical vein endothelial cells in normal rat AMs, and these activities were significantly inhibited by quinacrine. *In vivo,* rats with pancreatitis that were given quinacrine showed decreased concentrations of NO_2^- and NO_3^- in the bronchoalveolar lavage fluid, and the Pao_2, lung edema, and lung permeability were improved significantly.

Conclusion

PLA_2 induces AMs to release NO, which contributes to lung injury in acute pancreatitis. This lung injury was prevented by the administration of the PLA_2 inhibitor quinacrine.

In the early stage, acute pancreatitis is known to be associated often with acute lung injury. Twenty percent of deaths from acute pancreatitis occur within the first 24 hours after hospital admission, and 95% of those who die have associated acute lung injury.[1] However, the events that link acute pancreatitis to acute lung injury are not fully understood.

Reportedly, increased levels of pancreatic phospholipase A_2 (secretory PLA_2 type I [$sPLA_2$-I]) are detected in the systemic circulation and bronchoalveolar lavage fluid from patients with lung injury in acute pancreatitis.[2,3] Recently, it was revealed that another type of PLA_2 (secretory PLA_2 type II [$sPLA_2$-II]) activity was elevated in serum from patients with various inflammatory states such as acute pancreatitis,[4] adult respiratory distress syndrome, septic shock,[5] experimental endotoxemia,[6] and multiple organ failure.[7] In these clinical conditions, a high level of PLA_2 causes lung injury by damaging pulmonary surfactant and promoting platelet-activating factor or eicosanoid production that mediates direct or neutrophil-mediated tissue injury.[8,9] Further, serum PLA_2 activity correlated with serum $sPLA_2$-II level rather than with $sPLA_2$-I level, and lung and renal injury in acute pancreatitis was associated with serum $sPLA_2$-II elevation but not with $sPLA_2$-I elevation.[10–12]

Several investigators have reported that PLA_2 regulates the cytokine production of monocytes or macrophages and the phagocytosis and superoxide (O_2^-) generation of neu-

Correspondence: Takashi Morisaki, MD, Division of Clinical Immunology, Department of Surgery 1, Kyushu University, Faculty of Medicine, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan.
Accepted for publication July 14, 1998.

trophils.[13–15] Further, a PLA_2 inhibitor simultaneously reduced nitric oxide (NO) and O_2^- generation and cytotoxicity of rat cerebellar granule cells.[16] However, PLA_2-mediated NO induction of alveolar macrophages (AMs) in lung injury associated with acute pancreatitis has not been elucidated sufficiently.

In a previous study, we showed that AMs generate NO and O_2^- simultaneously and injure endothelial cells in lung injury of rats with acute pancreatitis.[17] In this study, we investigate whether PLA_2 induces NO production of AMs and mediates acute lung injury in pancreatitis.

MATERIALS AND METHODS

Chemicals

The following chemicals were purchased and used for the experiments: 1-2-dipalmitoyl-sn-glycerol-3-phosphorylcholine (DPPC), glycine, heptane, isopropanol, and octylphenyl ether (Triton X-100) were from Wako Pure Chemical Industries (Osaka, Japan). Toluene and Scintisol ALX-2 were from Dojindo Chemical Co. (Kumamoto, Japan). PLA_2 (type II) was from Takara (Shiga, Japan). Quinacrine and Evans blue were from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 medium and trypsin were from Sanko Chemicals (Tokyo, Japan), and fetal calf serum (FCS) was from Filtron, Australia.

Animal Model

Male Wistar King rats (Institute for Experimental Animals, Kyushu University Faculty of Medicine) weighing 250 to 300 g were fed standard laboratory chow and water *ad libitum*.

Acute pancreatitis was induced by selective pancreatic duct ligation (SPL), as previously described.[17] This rat model is particularly important because it closely resembles human acute pancreatitis induced by the mechanical obstruction of the main pancreatic duct during or after gallstone passage.[18] The rats were anesthetized with ether to undergo a midline laparotomy, and the duodenal portion of the pancreas was exposed. The pancreatic ducts, which flow into the common bile duct, were ligated with 7-0 nylon along the entire length of the common bile duct bilaterally. In our rat pancreatitis model, pancreatic damage is thought to be principally the result of edematous pancreatitis. We reached this conclusion for several reasons. Interstitial edema resulting in the separation of pancreatic lobules and acinar cells was observed 6 hours after pancreatic duct ligation. Enlarged edematous tissue and minimal parenchymal necrosis were seen, and numerous granulocytes were present in the interlobular connective tissue and around the necrotic foci 24 hours after duct ligation.

To study the effect of PLA_2 inhibition, quinacrine (5 mg/kg dissolved in 0.5 ml saline) was delivered intravenously for 45 minutes after SPL. Pancreatitis rats without quinacrine treatment were given the same volume of saline with the same timing. Sham control rats underwent studies after the same midline abdominal incision but not the SPL. In separate preliminary experiments, quinacrine administration did not influence the general condition and respiratory function of rats. All procedures involving rats were performed according to the guidelines described in the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

Alveolar Macrophage Isolation

Rats were anesthetized with intraperitoneal injections of sodium pentobarbital (50 mg/kg; Abbott Laboratories, North Chicago, IL). The rats were exsanguinated by transsecting their abdominal aortas, and bronchoalveolar lavage (BAL) was performed on rats killed 24 hours after the SPL (with or without quinacrine administration) and sham operation. Rats underwent five serial whole-lung lavages, with 10 ml of sterile normal saline per lavage, given through a tracheal cannula. An average of 42 ml of instilled saline was recovered from each rat.

The BAL fluid (BALF) was centrifuged at 280 g for 10 minutes at 4°C. The cell pellets were resuspended (1×10^6 cells/ml) in RPMI 1640 medium containing 10% heat-inactivated FCS. The cell suspension was then placed in plastic Petri dishes (Nunc, Roskilde, Denmark) and incubated at 37°C for 1 hour in a CO_2 incubator (5% CO_2 + 95% air; NAC Systems, Tualatin, OR). Nonadherent cells were removed from adherent macrophages by washing them with RPMI 1640 medium. Purified AMs were recovered by gently rubbing the dishes with a rubber policeman.

The recovery rate of AMs, determined by Giemsa staining, was >98%, and no platelet contamination was detected. Cell viability, assessed by trypan blue dye exclusion, consistently exceeded 96%. Lactic dehydrogenase concentration in the BALF was also measured as a marker of lung tissue injury using an autoanalyzer (Hitachi 7070, Tokyo, Japan). To determine NO level in the lung space, the concentration of NO_2^- and NO_3^- in the BALF was measured as described below.

PLA_2 Activity

Serum PLA_2 activity was measured by the Dole extraction system with a slight modification.[19] Blood sampling from the femoral artery was performed at various times (6, 12, 18, 24, and 48 hours after SPL), the blood samples were centrifuged at 1000*g* for 15 minutes, and the serum samples were isolated. The assay tubes were mixed with rat serum (100 μl) and substrate for a total volume of 500 μl with 80 mM glycine (pH 9.0), 5 mM $CaCl_2$, and 50% glycerol on ice. The substrate contained 100 μM DPPC, about 10,000 cpm of 1-palmitoyl PC (Japan Isotope Association, Tokyo, Japan), and 200 μM Triton-X 100. The assay tubes were incubated in a shaking water bath at 60°C for 40 minutes.

The enzyme reaction was stopped by adding 2.6 ml of Dole's reagent (isopropanol:heptane:0.5 M H_2SO_4, 400: 100:20, vol/vol/vol) and vortexing immediately. The free fatty acids in each heptane phase were extracted into a scintillation fluid and counted by a liquid scintillation counter (LSC-903, Aloka, Tokyo, Japan). The enzyme activity is expressed as the amount of hydrolyzed DPPC per minute.

NITRIC OXIDE PRODUCTION BY ALVEOLAR MACROPHAGES

NO production by AMs was assessed by measuring the accumulation of nitrite (NO_2^-) and nitrate (NO_3^-) in culture medium using a spectrometric assay based on the Griess reaction with sodium nitrite as the standard.[17,20] AMs from normal rats (5×10^5 cells/well in 0.5 ml of RPMI 1640 with 10% FCS) were cultured in a 24-well microplate (Nunc) with serum from pancreatitis rats (high-PLA$_2$ serum obtained 6 hours after SPL; 100 μl/ml) or with various concentrations of PLA$_2$ (0, 20, 50, 100 ng/ml) for 24 hours at 37°C in an incubator (5% CO_2 + 95% air). In other experiments, the PLA$_2$ inhibitor quinacrine (1 mM) or the NO synthase (NOS) inhibitor L-NMMA (50 μM) was added to the culture of AMs stimulated with PLA$_2$ (50 ng/ml). A 50-μl aliquot of culture supernatant was mixed with 50 μl of the Griess reagent after conversion of NO_3^- to NO_2^- by NO_3^- reductase in a 96-well microplate and incubated for 10 minutes at room temperature. The optical density was measured using an automatic plate reader (Easy Reader, SLT Lab Instruments, Grodig, Austria) at a wavelength of 570 nm, and the total NO_2^- + NO_3^- concentration was determined from a standard curve prepared with known concentrations of $NaNO_2$.

Amplification of Inducible NOS (iNOS) mRNA from AMs by Reverse Transcription–Polymerase Chain Reaction

We used reverse transcription–polymerase chain reaction (RT-PCR) to examine the effect of the PLA$_2$ inhibitor quinacrine on iNOS mRNA expression in AMs. AMs were isolated from normal rats as previously described and cultured in plastic Petri dishes (2×10^6 cells in 2 ml of RPMI 1640/dish) with PLA$_2$ (50 ng/ml) stimulation and with various concentrations of quinacrine (0, 5, 10, 20, 50, 100 μM). After incubation at 37°C for 1 hour in a CO_2 incubator (5% CO_2 + 95% air), AMs were harvested and total RNA was isolated from AMs as previously described[21] and quantified by spectrophotometry. RNA (1 μg) from each sample was reverse transcribed to cDNA using reverse transcriptase (SuperscriptII, GIBCO BRL, Gaithersburg, MD) and amplified by PCR with Taq polymerase (Takara, Kyoto, Japan).[17] The PCR primers for iNOS of rat were designed using a published sequence for rat vascular smooth muscle iNOS[22] (purchased from Funakoshi, Japan). The sense primer was 5′-GCA-TGG-AAC-AGT-ATA-AGG-CAA-ACA-3′, and the antisense primer was ′5-GTT-TCT-GGT-CGA-TGT-CAT-GAG-CAA-3′. The predicted length of PCR products amplified with these primers was 222 bp. PCR was carried out for 30 cycles at an annealing temperature of 60°C with β-actin used as internal control. PCR products were electrophoresed on a 1% agarose gel and visualized with ethidium bromide staining and ultraviolet transillumination.[23]

Preparation of Human Umbilical Vein Endothelial Cells (HUVECs) and Cytotoxicity Assay

Alveolar macrophage cytotoxicity was examined using ^{51}Cr release assay with HUVECs, as previously described.[17] Briefly, HUVECs were isolated from fresh, healthy umbilical cord by trypsin digestion and were grown to confluence during a second passage on 24-well, flat-bottom microplates at 37°C in a CO_2 incubator (5% CO_2 + 95% air).[24] HUVECs were labeled with sodium chromate (^{51}Cr; 75 kBq/well) during the last hour of this culture.

After three washings of the monolayers with RPMI 1640, AMs (5×10^5 cells) in RPMI 1640 supplemented with 10% FCS were added to the monolayers at a final volume of 0.5 ml/well and cocultured at 37°C in a CO_2 incubator (5% CO_2 + 95% air) for 18 hours. In some experiments, L-NMMA (50 μM) or PLA$_2$ (50 ng/ml, with or without addition of quinacrine, 1 mM) was added to the culture of AMs. Cytotoxicity of HUVECs by AMs was determined by quantifying ^{51}Cr release from the endothelial cells. Supernatant (0.5 ml) was removed from each well, and ^{51}Cr release was counted in a gamma counter (1282 Compugamma, LKB-Wallac, Turku, Finland). The percentage cytotoxicity was calculated using the following formula[25]:

% cytotoxicity = ([experimental release − spontaneous release])/([maximal release − spontaneous release]) × 100

Quantification of Lung Injury

Lung injury induced by SPL was quantitated by measuring the concentration of Evans blue dye in the lung after intravenous administration and lung weight 24 hours after the SPL operation, the time when lung function was worst in this model, as shown in our previous study.[17] Evans blue dye, which binds to albumin, has been used as a sensitive protein extravasation marker of inflammatory tissue injury models.[26,27] Evans blue dye (20 mg/kg) was injected intravenously before SPL or the sham operation. Twenty-four hours after surgery, the lungs and heart were excised, and the pulmonary vasculature was cleared of blood by gently infusing normal saline into the right ventricle. After weighing, the lungs were placed in 5 ml formamide, homogenized for 40 seconds, and incubated at 37°C for 16 hours. The dye concentration of the tissue suspension was measured by

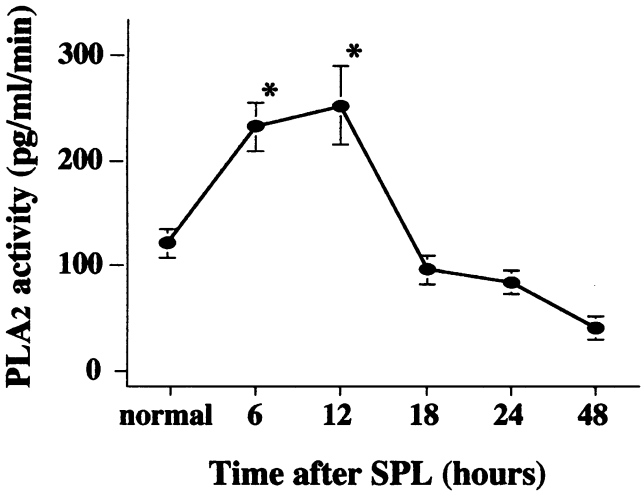

Figure 1. Changes in PLA_2 activity in rats with pancreatitis induced by SPL. Values represent mean ± SD. Significant differences from the untreated control rats are denoted as * $p < 0.001$.

spectrophotometry using an automatic plate reader (Easy Reader) at a wavelength of 620 nm. The Evans blue dye extraction from the lung was expressed as dye (ng)/wet lung weight (mg). In a separate group of animals not injected with Evans blue dye, the lungs were excised and measured at whole lung weight (wet lung weight). Then, the lungs were dried in an incubator at 120°C for 2 hours and measured at dry lung weight. Lung edema was expressed as wet lung weight (mg)/ dry lung weight (mg). As another index of lung dysfunction, the arterial blood oxygen pressure (Pao_2) was measured 24 hours after SPL with an autoanalyzer (ABL2 Acidbase Laboratory, Radiometer, Copenhagen, Denmark).

Statistical Analysis

All data are expressed as mean ± SD. Comparisons among multiple experimental groups and between each time point were analyzed using ANOVA. $P < 0.05$ was considered to be statistically significant.

RESULTS

Serum PLA_2 Activity in Rats After SPL

Serum PLA_2 activity rose rapidly, peaking 6 hours ($p < 0.001$) after SPL, and then decreased to normal level by 24 hours after SPL (Fig. 1). In this pancreatitis model, iNOS mRNA also was induced in AMs at 6 hours after SPL with histologic evidence of lung injury and depletion of Pao_2, which were caused by the large amount of NO production from activated AMs, as shown in our previous study.[17]

NO Production of AMs Stimulated with PLA_2

To study whether PLA_2 induces NO production of AMs, we measured $NO_2^- + NO_3^-$ concentration in culture media

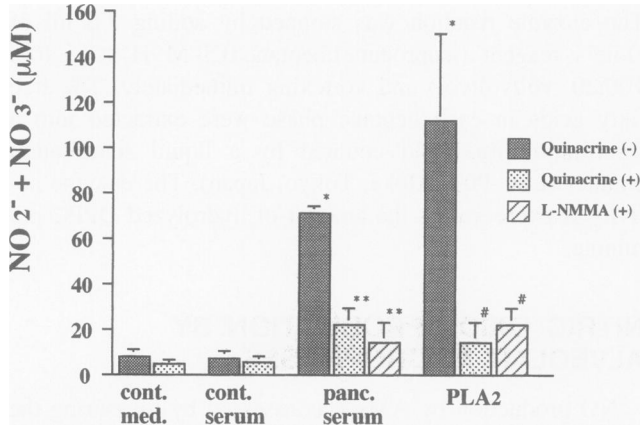

Figure 2. NO generation from AMs stimulated by the serum from rats with pancreatitis or PLA_2 with or without the addition of NO inhibitor L-NMMA and/or PLA_2 inhibitor quinacrine. AMs were stimulated by control medium, control serum, high-PLA_2 serum, and PLA_2. * $p < 0.01$ *vs.* control medium and control serum; ** $p < 0.01$ *vs.* high-PLA_2 serum without quinacrine and L-NMMA; # $p < 0.01$ *vs.* PLA_2 without quinacrine and L-NMMA.

of AMs stimulated with high-PLA_2 serum from rats (6 hours after SPL) and PLA_2 (50 ng/ml), with or without addition of the PLA_2 inhibitor quinacrine. As shown in Figure 2, both PLA_2 and high-PLA_2 serum induced normal rat AMs to produce large amounts of $NO_2^- + NO_3^-$ ($p < 0.01$), and these elevations were inhibited significantly by quinacrine ($p < 0.01$). In addition, normal AMs stimulated by PLA_2 or high-PLA_2 serum produced large quantities of $NO_2^- + NO_3^-$ ($p < 0.01$), and these levels were inhibited by NOS inhibitor L-NMMA (50 μM; $p < 0.01$; see Fig. 2). These results indicate that $NO_2^- + NO_3^-$ concentration in AM culture media reflects NO production from AMs.

Figure 3 shows $NO_2^- + NO_3^-$ production by AMs stimulated with various concentrations of PLA_2. $NO_2^- + NO_3^-$ production by AMs increased dose-dependently with the addition of PLA_2 ($p < 0.01$ *vs.* 0 ng/ml).

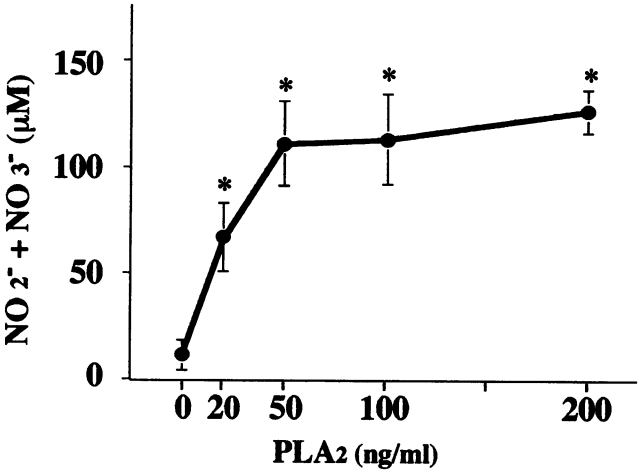

Figure 3. NO generation from AMs stimulated by various concentrations of PLA_2. AMs stimulated by PLA_2 produced threefold more $NO_2^- + NO_3^-$ than did AMs not stimulated (* $p < 0.01$ *vs.* 0 ng/ml).

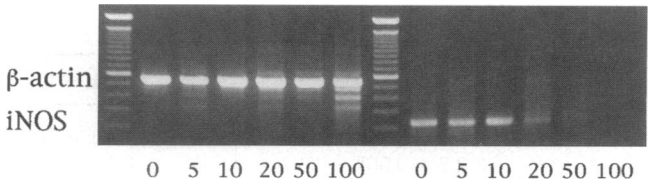

Figure 4. Effect of PLA$_2$ inhibitor quinacrine on iNOS mRNA expression in AMs stimulated by PLA$_2$ (50 ng/ml). iNOS mRNA expression was inhibited by various concentrations of quinacrine (0, 5, 10, 20, 50, 100 μM) examined by RT-PCR. β-actin was used as a positive control. The expected product size for the iNOS primer was 222 bp.

Expression of iNOS mRNA in AMs Stimulated by PLA$_2$

To ascertain whether the increased NO production was derived from the NOS induced in AMs by stimulation with PLA$_2$, we examined iNOS mRNA expression in AMs by RT-PCR. As shown in Figure 4, we detected iNOS expression in AMs stimulated with PLA$_2$ as the amplified product of a predicted length (222 bp). However, when AMs were pretreated with the PLA$_2$ inhibitor quinacrine, iNOS expression in PLA$_2$-stimulated AMs was reduced dose-dependently and was not detected with 100 μM of quinacrine. β-actin, used as internal control, was detected in all samples. These results suggest that iNOS was induced in the AMs with PLA$_2$ stimulation and that this iNOS mRNA expression was inhibited by quinacrine.

Cytotoxicity of AMs Against HUVECs

In this pancreatitis model, alveolar wall thickening caused by edema was observed histologically.[17] This edema formation was caused by endothelial cell damage and changes in microvascular permeability.[28] To determine the capacity of AMs stimulated with PLA$_2$ to cause endothelial damage, we examined the cytotoxic activity of AMs against HUVECs using a ^{51}Cr release assay (Fig. 5). When HUVECs were coincubated with AMs, the addition of PLA$_2$ enhanced the endothelial cell injury to fourfold of that without PLA$_2$ stimulation ($p < 0.01$). This cytotoxicity was reduced by the addition of NO inhibitor L-NMMA or PLA$_2$ inhibitor quinacrine ($p < 0.01$). However, the same concentration of PLA$_2$ (without AMs coculture) had no cytotoxic effect. These results suggest that AMs activated by PLA$_2$ injure endothelial cells and that NO derived from AMs is responsible for this cytotoxicity.

In Vivo Effect of the PLA$_2$ Inhibitor Quinacrine on Lung Injury

To examine the effect of a PLA$_2$ inhibitor on acute lung injury associated with acute pancreatitis, we injected the PLA$_2$ inhibitor quinacrine (5 mg/kg dissolved in 0.5 ml saline) into the pancreatitis rats (Table 1). After injection of quinacrine, the NO_2^- + NO_3^- concentration measured in

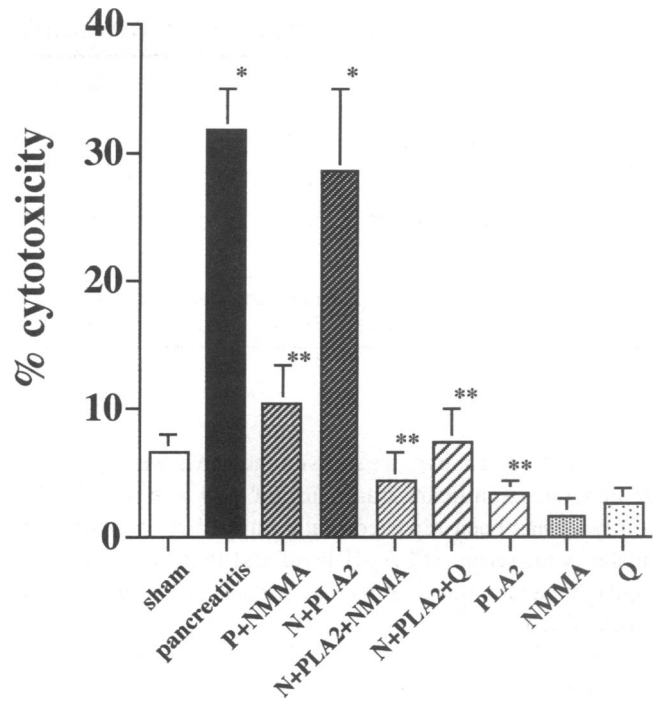

Figure 5. Cytotoxic reaction of AMs to HUVECs examined by ^{51}Cr release assay. HUVECs were coincubated with AMs from sham-operated control rats, SPL-operated control rats, or normal control rat, with or without addition of PLA$_2$, L-NMMA, or quinacrine. Sham, sham-operated control rat; normal & N, AMs of normal rat; pancreatitis & P, AMs of SPL-operated pancreatitis rat; PLA$_2$, addition of 50 ng/ml PLA$_2$ to culture medium; NMMA, addition of 50 μM NOS inhibitor L-NMMA to culture medium; Q, addition of 1 mM PLA$_2$ inhibitor quinacrine. * $p <$ 0.01 *vs.* sham control. ** $p < 0.01$ *vs.* pancreatitis and N + PLA$_2$.

the BALF of pancreatitis rats 24 hours after SPL was decreased significantly ($p < 0.0001$) compared with pancreatitis rats without administration of quinacrine. The lactic dehydrogenase level in the BALF, a marker of lung injury, also was decreased in parallel ($p < 0.01$). Further, the administration of quinacrine drastically improved the Pa_{O_2} (as an index of lung function) in rats with pancreatitis to 99.0 ± 1.25 mmHg ($p < 0.0001$ *vs.* SPL without quinacrine, 72.7 ± 2.81 mmHg; sham control, 110.9 ± 3.38 mmHg), which was nearly as much improvement as that found in the Evans blue dye ratio and wet/dry lung weight ratio ($p < 0.0001$), which were indices of lung permeability and lung edema, respectively. These results suggest that the administration of quinacrine reduced NO production in alveolar spaces and resulted in less lung injury during the early phase of acute pancreatitis.

DISCUSSION

This study shows that PLA$_2$ induces iNOS expression and NO generation of AMs, which partially contribute to lung injury. We found that the PLA$_2$ inhibitor quinacrine inhibited iNOS expression in AMs and reduced lung injury in acute pancreatitis.

Table 1. *IN VIVO* EFFECTS OF QUINACRINE FOR LUNG INJURY IN PANCREATITIS RATS

	Sham	SPL	SPL + Q
NO_2^- + NO_3^- in BALF (μM)	44.5 ± 6.44	91.7 ± 8.26*	56.0 ± 5.37**
LDH in BALF (IU/l)	1.0 ± 0.82	2.8 ± 0.96#	1.3 ± 0.50##
PaO_2 (mmHg)	110.9 ± 3.38	72.7 ± 2.81*	99.0 ± 1.25**
Evans blue permeability ratio (%)	13.9 ± 1.6	23.9 ± 4.6*	16.8 ± 1.0#
Wet/dry lung weight ratio	11.5 ± 0.82	19.9 ± 2.4*	13.2 ± 0.5**

Sham: rats with sham operation; SPL: rats with SPL operation; SPL + Q: rats with administration of quinacrine after SPL LDH: lactic dehydrogenase.
* $p < 0.0001$ *vs.* sham, ** $p < 0.0001$ *vs.* SPL, # $p < 0.01$ *vs.* sham, ## $p < 0.01$ *vs.* SPL.

As mentioned above, we showed that AMs were activated to generate significant quantities of NO and O_2^-.[17] Recently, it has been reported that serum PLA_2 activity correlated with serum $sPLA_2$-II level and that lung and renal injury in acute pancreatitis are associated with elevated serum $sPLA_2$-II.[28,29]

It is well known that circulating PLA_2 activity causes lung injury by damaging alveolar surfactant or by reacting with cell membrane to release inflammatory mediators such as eicosanoids and platelet-activating factor, which mediate direct tissue injury or induce neutrophil-mediated tissue injury. However, the results from our current model indicate that AMs appear to play a major role in the development and progression of lung injury because the accumulation of neutrophils in the alveolar spaces was not shown so prominently in our previous study.[17] Therefore, we hypothesized that increasing serum PLA_2, especially type II, induces iNOS in AMs and mediates lung injury in pancreatitis rats. In this model, serum PLA_2 activity peaked from 6 to 12 hours after SPL, iNOS mRNA of AMs was induced concomitantly,[17] and AMs incubated with this activated-PLA_2 serum generated a large amount of NO. Next, we showed that AMs stimulated with type II PLA_2 were activated to generate large amounts of NO in a dose-dependent manner and to express iNOS mRNA. This iNOS mRNA expression in AMs was inhibited dose-dependently by the PLA_2 inhibitor quinacrine. These results indicate that elevated serum PLA_2 activity contributes to inducing NO generation from AMs in rats with acute pancreatitis.

This speculation is supported by several studies. Recently, Kurose et al[30] reported that an increased production of NO in rat Kupffer cells was preceded by activated NF-kappaβ, which moved into the nuclei, and quinacrine significantly attenuated the increase in NF-kappaβ activation and NO production.[30] Further, in a study of an animal model of inflammation, when the rat air pouch was stimulated with zymosan, levels of NO_2^-/NO_3^-, secretory PLA_2 in exudates, and NOS activity in polymorphonuclear leukocytes and monocytes increased. Also, treatment of the animals with the PLA_2 inhibitor dexamethasone inhibited cellular NOS activity (*i.e.*, NO_2^-/NO_3^- levels).[31]

Next, we examined the cytotoxicity toward HUVECs by activated AMs with $sPLA_2$-II because change in microvascular permeability caused by endothelial cell damage is the major factor in lung edema.[28] AMs stimulated with $sPLA_2$-II cocultured on a HUVEC monolayer caused marked damage to the vascular endothelial cells. In contrast, the administration of the NO inhibitor L-NMMA or the PLA_2 inhibitor quinacrine significantly inhibited endothelial damage caused by AMs activated by $sPLA_2$-II. However, the same dose of $sPLA_2$ alone did not have cytotoxicity toward HUVECs. These results suggest that acute lung injury in this model was mediated by the cytotoxic effects of NO derived from $sPLA_2$-stimulated AMs, probably by the existence of O_2^- in the endothelial cells, but not by a direct effect of PLA_2 itself or its reactive inflammatory mediators. Our suggestion is supported by some reports that NO and O_2^- simultaneously generated by AMs produce peroxynitrite or its toxic intermediates, which damage endothelial cells and/or increase microvascular permeability, causing acute lung injury.[32,33]

Further, we examined the effect of PLA_2 inhibition for preventing lung injury associated with pancreatitis and found that administration of the PLA_2 inhibitor quinacrine 45 minutes after SPL lessens the level of acute lung injury in the early stage of acute pancreatitis in a rat model. After the administration of quinacrine, NO levels in the lung space decreased concomitantly with the improvement of lactic dehydrogenase levels in BALF and the reduction of lung edema, examined by lung weight and permeability of Evans blue in the lung tissue. These results indicate that PLA_2 mediates lung injury associated with NO elevation in the lung space. Also, AMs activated by PLA_2 may contribute partially to this lung injury by NO elevation, because AMs stimulated with PLA_2 caused marked damage to the vascular endothelial cells, whereas the same dose of PLA_2 alone did not have cytotoxic effects on the endothelial cells.

In addition, we have measured NO production from Kupffer cells in this model, but it was <60% of NO production from AMs, and the increased NO level was observed primarily in the alveolar space. Therefore, we think this cytotoxic NO may be derived from AMs, not from Kupffer cells.

Some investigators have reported that PLA_2 activities are increased in patient serum in acute pancreatitis, sepsis, peritonitis, and multiple injury, and that increased PLA_2 type II values are found in conditions involving inflammation of these diseases,[34] or that serum PLA_2 activities correlate well with the severity of acute pancreatitis.[35] In patients with acute edematous pancreatitis, serum PLA_2 activity rose during the first 3 days from onset of pancreatitis and rapidly decreased on the fourth day, and the mean PLA_2 activity in these patients was three to five times greater than that in the controls.[34,35]

The NO production process and the role of NO in human acute pancreatitis are still unclear. In patients with septic shock, which is often associated with severe pancreatitis, a significant rise in neutrophil was observed concomitant with plasma NO increase.[36] In animal models of acute pancreatitis, an NO donor reduced pancreatic edema and necrosis and conferred protection against trypsinogen activation, which correlated with death.[37] In contrast, NO contributed to lung injury in the early phase of acute pancreatitis.[17] We think a PLA_2 inhibitor has a beneficial effect on both pancreatic and lung injury in acute pancreatitis. Quinacrine, a PLA_2 inhibitor, has been used as treatment for malaria in humans. In addition, quinacrine reportedly inhibited superoxide generation from human neutrophils.[38] These results suggest that quinacrine can be effective for human macrophages. Thus, we believe quinacrine can be used therapeutically in human pancreatic disease.

In conclusion, AMs are activated by PLA_2, mainly by $sPLA_2$ type II, and produce a large amount of NO that contributes to lung injury in acute pancreatitis. Because the lung injury was reduced by the administration of the PLA_2 inhibitor quinacrine during the early stage of acute pancreatitis, blocking of PLA_2 activity may be beneficial for preventing acute lung injury associated with pancreatitis.

References

1. Renner IG, Savage WB. Pantoja JL, Renner VJ. Death due to acute pancreatitis. A retrospective analysis of 405 autopsy cases. Dig Dis Sci 1985; 30:1005–1018.
2. Edelson JD, Vadas P, Villar J, et al. Acute lung injury induced by phospholipase A_2. Structural and functional changes. Am Rev Respir Dis 1991; 143:5(pt 1):1102–1109.
3. Nordback I, Teerenhovi O, Auvinen O, et al. Human pancreatic phospholipase A_2 in acute necrotizing pancreatitis. Digestion 1989; 42:128–134.
4. Offenstadt G, Pinta P, Masliah J, et al. Phospholipase and prophospholipase activities in bronchoalveolar lavage fluid in severe acute pulmonary disease with or without ARDS. Intensive Care Med 1981; 71:285–1290.
5. Buchler M, Malfertheiner P, Schadlich H, et al. Role of phospholipase A_2 in human acute pancreatitis. Gastroenterology 1989; 97:1521–1526.
6. Vadas P, Pruzanski W. Induction of group II phospholipase A_2 expression and pathogenesis of the sepsis syndrome. Circ Shock 1993; 39:160–167.
7. Santos AA, Browning JL, Scheltinga MR, et al. Are events after endotoxemia related to circulating phospholipase A_2? Ann Surg 1994; 219:183–192.
8. Nyman KM, Uhl W, Forsstrom J, et al. Serum phospholipase A_2 in patients with multiple organ failure. J Surg Res 1996; 60:7–14.
9. Zhou W, McCollum MO, Levine BA, Olson MS. Role of platelet-activating factor in pancreatitis-associated acute lung injury in the rat. Am J Pathol 1992; 140:971–979.
10. Nevalainen TJ. Serum phospholipase A_2 in inflammatory diseases. Clin Chem 1993; 39:2453–2459.
11. Das SK, Scott MT, McCuiston S. Effects of experimental acute pancreatitis in dogs on metabolism of lung surfactant phosphatidylcholine. Biochem Biophys Res Commun 1987: 145:612–618.
12. Anderson BO, Moore EE, Banerjee A. Phospholipase A_2 regulates critical inflammatory mediators of multiple organ failure. J Surg Res 1994; 56:199–205.
13. Bomalaski JS, Ford T, Hudson AP, Clark MA. Phospholipase A_2-activating protein induces the synthesis of IL-1 and TNF in human monocytes. J Immunol 1995; 154:4027–4031.
14. Weiss J, Inada M, Elsbach P, Crowl RM. Structural determinants of the action against *Escherichia coli* of a human inflammatory fluid phospholipase A_2 in concert with polymorphonuclear leukocytes. J Biol Chem 1994; 269:26331–26337.
15. Dana R, Malech HL, Levy R. The requirement for phospholipase A_2 for activation of the assembled NADPH oxidase in human neutrophils. Biochem J 1994; 297:217–223.
16. Gunasekar PG, Kanthasamy AG, Borowitz JL, Isom GE. NMDA receptor activation produces concurrent generation of nitric oxide and reactive oxygen species: implication for cell death. J Neurochem 1995: 65:2016–2021.
17. Tsukahara Y, Horita Y, Torisu M, et al. Role of nitric oxide derived from alveolar macrophages in the early phase of acute pancreatitis. J Surg Res 1996; 66:43–50.
18. Gukovskaya AS, Perkins P, Zaninovic V, et al. Mechanisms of cell death after pancreatic duct obstruction in the opossum and the rat. Gastroenterology 1996;110:875–884.
19. Sano M, Yokoyama M, Morishita T, et al. Elevation of the phospholipase A_2 activity in peritoneal fluid cells from women with endometriosis. Fertil Steril 1994; 61:657–662.
20. Ding A, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J Immunol 1988; 141:2407–2412.
21. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156–159.
22. Nunokawa Y, Ishida N, Tanaka S. Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. Biochem Biophys Res Commun 1993; 191:89–94.
23. Markewitz BA, Michael JR, Kohan DE. Cytokine-induced expression of nitric oxide synthase in rat renal tubular cells. J Clin Invest 1993; 91:2138–2143.
24. Morisaki T, Goya T, Toh H, et al. The anti Mac-1 monoclonal antibody inhibits neutrophil sequestration in lung and liver in a septic murine model. Clin Immunol Immunopathol 1991; 61:365–375.
25. Uchiyama A, Morisaki T, Torisu M. Evidence that induction and regulation of lymphokine-activated killer (LAK) activity are mediated by changes in tumour-binding potential of lymphocytes after activation by interleukin-2 (IL-2). Immunology 1991; 74:94–98.
26. Rogers DF, Boschetto P, Barnes PJ. Plasma exudation: correlation between Evans blue dye and radiolabelled albumin in guinea pig airways in vivo. J Pharmacol Methods 1989; 21:309–315.
27. Patterson CE, Rhoades RA, Garcia JGN. Evans blue dye as a marker of albumin clearance in cultured endothelial monolayer and isolated lung. J Appl Physiol 1992; 72:865–873.
28. Closa D, Bardaji M, Hotter G, et al. Hepatic involvement in pancreatitis-induced lung damage. Am J Physiol 1996; 270(pt 1):G6–13.

29. Formela LJ, Galloway SW, Kingsnorth AN. Inflammatory mediators in acute pancreatitis. Br J Surg 1995; 82:6–13.
30. Kurose I, Saito H, Miura S, et al. CD18/ICAM-1-dependent oxidative NF-kappaβ activation leading to nitric oxide production in rat Kupffer cells cocultured with syngeneic hepatoma cells. J Clin Invest 1997; 99:867–878.
31. Payá M, García Pastor P, Coloma J, Alcaraz MJ. Nitric oxide synthase and cyclo-oxygenase pathways in the inflammatory response induced by zymosan in the rat air pouch. Br J Pharmacol 1997; 120:1445–1452.
32. Lechin AE, Varon J. Adult respiratory distress syndrome (ARDS): the basics. J Emerg Med 1994; 12:63–68.
33. Shasby DM, Lind SE, Shasby SS, et al. Reversible oxidant-induced increases in albumin transfer across cultured endothelium: alterations in cell shape and calcium homeostasis. Blood 1985; 56:605–614.
34. Nevalainen TJ, Gronroos JM, Kortesuo PT. Pancreatic and synovial type phospholipase A_2 serum samples from patients with severe acute pancreatitis. Gut 1993; 34:1133–1136.
35. Makela A. Kuusi T, Schroder T. Serum phospholipase A_2, amylase, lipase, and urinary amylase activities in relation to the severity of acute pancreatitis. Eur J Surg 1997; 163:915–922.
36. Nelly IJ, Copland M, Haj M, et al. Plasma nitrate concentration in neutropenic and non-neutropenic patients with suspected septicaemia. J Haematol 1995; 89:199–202.
37. Werner J, Rivera J, Firnandez del Castillo C, et al. Differing roles of nitric oxide in the pathogenesis of acute edematous versus necrotizing pancreatitis. Surgery 1997; 121:23–30.
38. Dana R, Malech HL, Levy R. The requirement for phospholipase A_2 for activation of the assembled NADPH oxidase in human neutrophils. Biochem J 1994; 297:217–223.